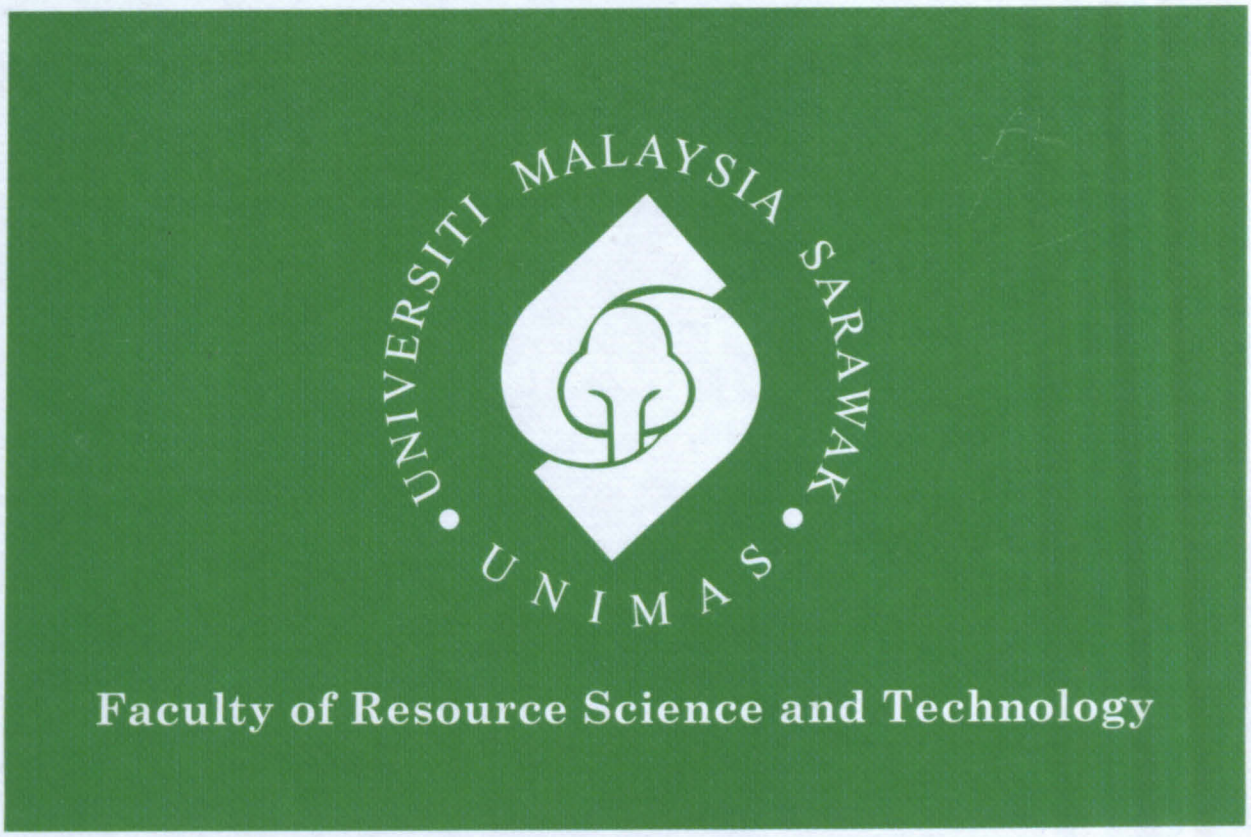

2D-PAGE AND BIOINFORMATICS ANALYSIS OF DISEASED AND NON-DISEASED *PIPER NIGRUM*

AHMAT AMRIN B. ABDUL MUTALIB
50353

Bachelor of Science with Honours
(Resource Biotechnology)
2018

UNIVERSITI MALAYSIA SARAWAK

Grade: _____

Please tick (√)

Final Year Project Report	√
Masters	
PhD	

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This declaration is made on the 11 day of June year 2018

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Profesor Madya Dr. Mohd Hasnain Md Hussain
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2D-PAGE ANALYSIS OF DISEASED AND NON-DISEASED *PIPER NIGRUM*

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A project report submitted in partial fulfillment of the Final Year Project (STF 3013)

Bachelor of Science with Honours Resource Biotechnology

Faculty of Resource Science and Technology

2018

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PhD ☐

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2d-Page and Bioinformatics Analysis of Diseased and Non-Diseased *Piper nigrum*

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ABSTRACT

Piper nigrum have been used widely documented in many sectors such as food and medicine industry. Plant as a role model shows various strategy to survive under different pressure. However, the information on proteins associate with the properties and biological process under environmental stress for *P. nigrum* are limited. The main aim for this study was to extract protein and differentiate the protein expressed through *in silco* analysis from diseased and non-diseased steam and root of *P. nigrum*. The study was iniatiated by extracting protein by using imidazole-tca precipitation method. The protein was quatified by using Bradford assay and further diffrentiated by its isoelectric point in 2D PAGE. The results were analyzed by the aid of Delta2D software and perform direct differentiation through the statistics. The protein content within each plant vary slightly as it were dependent on the pressure applied to the plant. A distinct spot difference expressed by the two different condition of the plant shows some protein to be suppressed while some protein were expressed more. The study provides an initial insight into the P. nigrum proteome and its response to disease.

Keywords: 2D PAGE, imidazole-tca precipitation, isoelectric point

ABSTRAK

Piper nigrum telah digunakan secara meluas dalam pelbagai sektor seperti industri makanan dan perubatan. Tumbuhan merupakan salah satu model yang baik dalam memaparkan pelbagai strategi untuk hidup dalam tekanan yang berbeza. Walau bagaimanapun, informasi terhadap protein-protein dengan sifat-sifat dan proses biologi terhadap tumbuhan tersebut adalah terhad. Tujuan utama kajian ini adalah untuk mengekstrak protein daripada P. nigrum dan menganalisa protein yang dinyatakan melalui in silco daripada batang dan akar yang berpenyakit dan tidak berpenyakit. Protein tersebut diekstrak menggunakan kaedah persipitasi imidazole-tca. Protein-protein terbesut diuji mengunakan Bradford dan selanjutnya dipisahkan kepada titik isoelektrik melalui 2D PAGE. Gel tersebut kemudian dianaliss dengan perisian Delta2D dan perbezaan telah dipaparkan dalam bentuk statistic. Setiap kandungan protein dalam setiap tumbuhan mempunyai perbezaan bergantung kepada tekanan yang dialami oleh tumbuhan tersebut. Hasilnya, terdapat perbezaan dinyatakan dari segi level protein daripada dua keadaan sampel yang berbeza. Gambaran gel menunjukan beberapa protein diekpresikan dalam quantity yang sedikit sementara yang lainnya diekpresikan lebih. Kajian ini memberi gambaran awal tentang proteom P. nigrum dan tindak balasnya terhadap penyakit.

Kata kunci: *2D PAGE, pemendakan imidazole-tca, titik isoelektrik*

Contents

LIST OF ABREVATION

2-DE - Two-dimensional electrophoresis

2-D - Two-dimensional

TCA - Trichloroacetic acid

SDS - Sodium dodecyl Sulfate

2-ME - 2-mercaptoethanol

IEF - isoelectric focusing

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CHAPTER 1: Introduction

Piper nigrum were plants that abundantly found in southeast Asia, a climber type of plant that has vine towards a pole (Gopalakrishnan et al., 1993). *P. nigrum* was commonly known as black pepper as its functional attributes for seasoning and traditional medicine (Ravindran, 2003).

The black pepper had abundant of a bioactive compound that was still in research that may contribute to the medicinal field (Suresh et al., 2007). The plant incorporates major of the plant overall protein including bioactive compounds used for medicinal purposes such as antioxidant (Gülçin, 2005). When the plant is under stress condition, some proteins were altered to regulate the signal transduction to manipulate the plant metabolism (Bohnert et al., 1995).

When a plant was wounded, the gene in the plant cell will produce a protein to give the signal to other cells for producing antibody to fight against invading organism. In some cases, the cell will activate defense mechanism such as ethylene production to removes the infected were mainly involving leaves and fruits (Ke & Saltveit, 1989). Protein role in the defense mechanism was mainly enzyme and cell signaling molecule regulating hormone production for the appropriate task (Kimmerer & Kozlowski, 1982).

The abilities of various species of *P. nigrum* being resistance towards the common disease were widely known. Thus, apprehend the functional protein presence within its healthy and wounded condition will help in understanding the plant development. To analyze the protein activity, it needs to be characterized for its role in biological processes. Subsequently, its essential to manipulate the plant condition when sampling as a different condition of plant regulate different protein concentration (Chandler & Robertson, 1994).

CHAPTER 1.1: Objectives

Protein from diseased and non-diseased of stem and root of *Piper nigrum* can be extracted by imidazole-tca method and further isolated by 2D-PAGE and analysed by Delta2d software. This study were conducted by using such method to achieve the following objective below.

To extract protein from stem and roots of diseased and non-diseased *Piper ngirum.*

To differentiate protein expressed between diseased and non-diseased *P. ngirum.*

To characterize the proteins through *in silico* analysis and relate to plant development.

CHAPTER 2.0: Literature Review

2.1 Protein

Protein is identified as a chain of the molecule attached to perform a specific task such as catalyst, transporting molecule, and even blocks for the cellular building of cells (Jeong et al., 2001). Abundant set of protein was categorized as proteome that expressed the organism's genome in either cell, tissue or whole organism level (Consortium, 2011). Protein can bind into a substrate and act as either inhibitor, cell signaling, and catalyst. As an inhibitor, it can inhibit the production of other protein, selectively bind towards targeted viral protease to block the requisite protein precursor for infectious viral particles production (Angiolillo et al., 1995).

Aside from that, it can interact with another molecule to act as a signaling molecule such as insulin, inducing the conversion of glucose to glycogen in the liver (Khan & Pessin, 2002). This is essential to regulate the glucose level in the blood. When the glucose level is low, hormonal protein such as glucagon is secreted to regulate the blood sugar level. Were secreted to regulate the blood sugar level.

In the plant, protein is synthesis as mainly as a defensive maneuver against pathogens and harmful organism. In stress condition by a phytopathogenic microorganism, the plant will synthesize inhibitory peptides to suppress the enzyme activities. First plant defensins that were reported were thionin in which cell membrane were altered its permeability from unwanted substances (Epple et al., 1997).

2.2 Black pepper (*Piper nigrum)*

Piper nigrum commonly known as black pepper is a family of Piperaceae that native to southeast Asia and also known as black gold for its interchange value between Europe and Southeast Asia country(Ravindran, 2003). It is used as a spice and seasoning in cooking while some were used as traditional medicine. *Piper nigrum* fruit is green in color and turn black when dried up. The white pepper was resulted in peeling off the outer skin of the fruit after pectin fermentation.

Piper nigrum is a plant that has a vast of functional protein aside from its chemical composition that shows piperine as high demanding effect such as anti-cancer, anti-depressant, anti-asthmatics (Bano et al., 1991). Protein associated with piperine is yet completely known, and the part of the protein can be extracted with today method. Protein such as piperine can increase the efficiency of drug absorption, and the bioactive compound can be extracted and separated by using total protein plant extraction method.

2.3 Protein extraction

Protein extraction have a vast technique available while some have the advantages over the other and all the technique have its limitation. Although extraction buffer is commonly used, it is more efficient when to combine with physical lysis by grinding through the cells into a fine power mixing with the buffer (Hopkins, 1991).

Protein can be extracted by using multiple techniques such as detergent chemicals, by using low ionic salt and shear force, all have the purpose of weakening the outer membrane of the cell. Low ionic salt method and detergent method were

commonly used due to their purification and efficiency reliability (Helenius & Simons, 1975).

Since protein is very sensitive toward temperature, the working condition is kept at a low temperature of 4°C while some may add a buffer for precaution purpose as protein were easily denatured. Most of the protein extraction required the uses of reducing agent such as 2-Mercaptoethanol to keep the protein from oxidizing so that the condition can be preserve (Brennan & O'neill, 1996). The purification of protein needed to be check by using assay. The commonly used protein assay is the standard protein assay to assess the absorbance value to a standard curve (Peterson, 1977). The protein value is calculated when diluted for the assay, and the good result should be near to the calculated value.

2.4 SDS PAGE

SDS stands for sodium dodecyl sulfate (SDS), and PAGE is polyacrylamide gel electrophoresis (PAGE). The technique separates macromolecule in an electric field by using polyacrylamide as the support medium while the SDS denature the proteins. Polypeptide chain can be bind to SDS based on its molecular masses (Takagi et al., 1975). The higher the mass, the slower the molecule will travel across the polyacrylamide gel. The polyacrylamide gel is meant to be restraining medium to restrict the movement of larger molecule migrate faster than the smaller molecule. The gel has a uniform density that able to separate charged molecule based on it molecular mass through electrophoresis (Segrest et al., 1971). The migration length will be negatively proportional to the log of its mass thus enable the approximate calculation of the unknown protein mass. The methodology employs by the Laemmli method were the standard ones that were used today, and slight changes to the method were based on the user requirement (Laemmli, 1970). The system commonly will cast

two layers of acrylamide in which the one for separation and the other layer will stack the moving protein together so that the sample protein can be compressed in the gel layer.

2.5 Two-dimensional electrophoresis

2-DE (Two-dimensional electrophoresis) were also using an electric field to separate the protein molecule. The advantages of 2-DE were that the gel can be ran with SDS PAGE and run through 2-dimensional analysis software to check the protein that is a presence (O'Farrell, 1975). It also can separate non-protein molecule by denaturing DNA (deoxyribonucleic acid) using DNA intercalator such as ethidium bromide from the supercoiled state.

2-DE by Bio-Rad uses SDS PAGE as the second dimension and using isoelectric focusing (IEF) as the first dimension (Washburn et al., 2001). IEF were used to separate the protein by ion while the second dimension were to separate protein by molecular weight. Coomassie Brilliant Blue is used as staining to detect the protein by staining the protein in spots (Neuhoff et al., 1988). The first layer of IEF and the second layer of SDS PAGE in 2DE will be stacked and run under image scanner so that it can be quantified by 2D gel analysis software. The spots of which the two-layer stack together will be shown by the software and represent protein presence in the sample.

2.6 Delta2D

Delta2D by DECODON had the most recent method that greatly help spot detection and management. In the version 4.7, the software includes a better improvement in which the software provides an automatic Ai detection and warping to overlay the same spot on different gel altogether. To help its user, version 4.7 were

made user friendly in which a click from suggested procedure could do all the work without much refinement.

CHAPTER 3: Materials and Methods

3.1 Sample Preparation

The sample of stem and root of Piper Nigrum were collected from Kampung Duyoh located at 1°20'27.6"N 110°01'33.6"E. In this study, only the stem and root part of the Piper Nigrum were used. Approximately 2 g of the sample was place in a cold sterile mortar and pestle. The sample was quickly disrupted by using pestle and crushed until it become powdery with the aid of liquid nitrogen.

3.2 Protein Extraction

3.2.1 TCA Extraction

The extraction was done based on slight modification of Nakamura et al. (2012). Each sample were transferred into 15 mL centrifuge tube then added with ice-cold extraction solution (50 mM imidazole-HCl, pH 7.4, 8 mM MgCl2, 50 mM 2-meracpthoethanol, and 12.5 % (v/v) glycerol) of ratio 1:3 at 4°c. Each tube was divided into 1.5ml centrifuge tube equally and were centrifuge at 16,000 g at 4°c for 10 minutes. The supernatant was collected into several sterile 1.5ml microcentrifuge tube added with equal volume of 20% TCA/acetone and left overnight for precipitation at freezer -20°c. The mixture was then centrifuged at 16,000 g at 4°c for 10 minutes to form pellet. The supernatant was discarded, and the leftover pellet were collected into single 1.5ml centrifuge tube. The pellet was wash with 1ml ice cold absolute acetone. This step is repeated 3 times. The pellet was then air dried and stored in the freezer -20°c.

3.3 Quantification

3.3.1 Bradford assay

Protein concentration was quantified by using Bradford protein assay (Bradford, 1976). The pellet samples were dissolved in 250 µl solubilized buffer. The sample dilutions were prepared in 1000 x, 500 x, 200 x and 100 x. Bovine serum albumin (BSA) was pipetted in volumes of 10, 20, 40, 60, 80 and 100 µl of 100 mg/mL y-globulin standard solution into the 1.5 ml tubes then filled up with ultrapure water until it reached 100 µl as a standard. 100 µl of distilled water was used as reagent blank. 1 ml of Bradford reagent (Coomassie Brilliant Blue G-250, 4.7% methanol, 10% phosphoric acid) was added into the sample dilutions and vortex until it mixed well. The spectrophotometer was set into A595 then the samples were measured, and the data was recorded in tabulate form.

3.4 SDS Page

3.4.1 SDS PAGE

The polyacrylamide gel was prepared according to the standard protocol (Laemmli, 1970). The sample were prepared with sample buffer (bromothymol blue, HCl, glycerol, sodium dodecyl sulfate(SDS), 2-mercaptoethanol, deionized water):sample, 1:4. The samples were loaded 10 µl into the well with SDS running buffer (15 g Tris, 72 g Glycine, 5 g SDS in 1 litre of distilled water). Then, the gel was run for 1 hour and 30 minutes at 120 V. The gels were stained with Coomassie blue G-250 stain and placed in a plastic container. The gel was shake overnight at platform shaker at 70 rpm. The remaining solution of Coomassie blue was pour off into recycle bottle. The gels were washed with ultrapure water for 10 minutes repeated for 2 times. Destain solution (10% ethanol, 2% phosphoric acid, ultrapure)

was added into the gel and shake for 20 minutes on platform shaker at 70 rpm. The gels then washed with ultrapure water for 10 minute and the results were analyzed by observing their bands on the gel.

3.4.2 2-Dimensional SDS page

Isoelectric focusing (IEF) method is followed according to IEF – 2DE protocols (Wu X. et al., 2013). The sample was mixed with rehydration buffer and incubated at room temperature for 1 hour. The sample of 125 ul with concentration of 1.67ug/ul was loaded into the 7cm IEF strip and incubate for 1 hour.

The IEF strip was then transferred to ceramic strip holder and overlay with 2.5 ml of mineral oil. The samples were run for 12 hours active rehydration. The IEF strip then transferred into another lane with addition of wick to the both end of each strip. Another 2.5ml of mineral oil was added and the samples was running for 5 hours (50 volt, 20°c focus temp). Excess mineral was then remove and equilibrated with overlay on the strip, 2.5 ml equilibration buffer 1 and equilibration buffer 2 for 10 minutes each respectively on platform shaker 70 rpm. The strip was removed from equilibration buffer 2 and dip into SDS running buffer. The strip was transferred onto the prepared SDS gel with an addition of overlay gel. The glass plate was then casted into the container with addition of SDS running buffer and ran about 1 hour and 30 minutes at 120 V. The gel was stained with Coomassie blue G-250 overnight. Then the gel was washed with ultrapure water 10 minutes each for 2 times on the platform shaker at 70 rpm. The destain solution was then added and shake for 20 minutes, 70 rpm. Then, the image of gel was captured by using Bio-Rad machine at Faculty Medicine, University Malaysia Sarawak under tif format.